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RESEARCH ARTICLE

Multiplicity of infection of *Plasmodium knowlesi* in Malaysia: an application of *Pkmsp-1* block IV

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ABSTRACT

In Malaysia presently, the main cause of human malaria is by the zoonotic monkey parasite *Plasmodium knowlesi*. A previous study has suggested that the *P. knowles*i merozoite surface protein 1 (*Pkmsp-1*) block IV to be a suitable multiplicity of infection (MOI) genotyping marker for knowlesi malaria. This study therefore aimed to investigate the usefulness of *Pkmsp-1* block IV in assessing the MOI of *P. knowlesi* in clinical isolates from Malaysia. Two allele-specific PCR primer pairs targeting the two allelic families of block IV (T1 and T2) were designed, and used to genotype *P. knowlesi* in 200 blood samples (100 from Peninsular Malaysia and 100 from Malaysian Borneo). Results showed that the mean MOI in Malaysian Borneo was slightly higher as compared to Peninsular Malaysia (1.58 and 1.40, respectively). Almost half of the total blood samples from Malaysian Borneo (52%) had polyclonal infections (i.e., more than one allele of any family type) as compared to Peninsular Malaysia (33%) samples. The T1 allelic family was more prevalent in Peninsular Malaysia (n=75) than in Malaysian Borneo (n=60). The T2 allelic family, however, was more prevalent in the Malaysian Borneo (n=87 *vs* n=53 respectively). This study shows that the single locus *Pkmsp-1* block IV can serve as a simple alternative genetic marker for estimating knowlesi malaria MOI in a population. Future MOI studies should focus on macaque populations as macaques are the natural host of *P. knowlesi*.

Keywords: *Plasmodium knowlesi*; merozoite surface protein 1; block IV; multiplicity of infection.

INTRODUCTION

For many years, human malaria in Malaysia has been predominantly attributed to infection with *Plasmodium falciparum* and *Plasmodium vivax*. However, zoonotic infections caused by the simian malaria parasite *Plasmodium knowlesi* have increased sharply in the past two decades. For instance, no indigenous human malaria case was officially reported in Malaysia in 2017-2021, but a total of 17,125 cases of zoonotic human knowlesi malaria have been reported since 2017 (World Health Organization, 2022). Every country in the Southeast Asia region has reported cases of this zoonotic malaria infection except Timor Leste (Vythilingam *et al*., 2018).

The natural macaque hosts of *P. knowlesi* are widely distributed in South-East Asia along with the *Anopheles* mosquito vectors of the Leucosphyrus Group (Moyes *et al*., 2016). The World Health Organization has reported that *P. knowlesi* infection is a public health concern among those who frequent or work in the forests (World Health Organization, 2017). Travellers to forests in South-East Asia, too, have risk of acquiring the infection (Müller & Schlagenhauf, 2014). Asymptomatic cases of *P. knowlesi* malaria have been reported and this may hamper national malaria elimination efforts (Fornace *et al*., 2016; Jiram *et al*., 2019; Noordin *et al*., 2020).

The multiplicity of infection (MOI) or interchangeably termed as the complexity of infection (COI) is defined as the number of genetically distinct genotypes of the same parasite species co-infecting an individual (Kolakovich *et al*., 1996). MOI is known to be a good surrogate predictor of transmission intensity as well as an important index to assess the efficacy of malaria intervention and control programs (Abukari *et al*., 2019). A high mean MOI is a reflection of a high transmission intensity within a population and vice versa (Abukari *et al*., 2019; Saleh Huddin *et al*., 2019). Microsatellite markers have been employed previously to ascertain the MOI of *P. knowlesi* infection in Malaysia (Divis *et al*., 2015, 2017; Saleh Huddin *et al*., 2019). However, there is no study reporting the use of *P. knowlesi* merozoite surface protein-1 *(Pkmsp-1)* in assessing MOI despite many studies have been reported for *P. falciparum* and *P. vivax* MOI (Snounou *et al*., 1999; Atroosh *et al*., 2011; Zhong *et al*., 2018).

The *Pkmsp-1* gene is made up of five conserved blocks (I, III, V, VII, and IX) that are intercalated by four polymorphic blocks (II, IV, VI, and VIII). Recently, a genetic diversity analysis on the polymorphic blocks revealed block IV as the most polymorphic with the highest insertion–deletion (indel) rates. Furthermore, two distinct allelic family types (T1 and T2) were identified within this block, thereby highlighting this block as a promising genotyping marker for MOI study of *P. knowlesi* malaria (Noordin *et al*., 2023). The present study aimed to investigate the usefulness *Pkmsp-1* block IV in assessing MOI of *P. knowlesi* among clinical isolates from Malaysia.

MATERIALS AND METHODS

Ethical approval

Ethical approval for human blood collection was granted by the Medical Research Subcommittee of the Malaysian Ministry of Health (NMRR-15-67223975) and written consent was obtained from each participant in the study.

Blood sample collection

The sample size was estimated following the approach of Schneider (2018), who used systematic simulation in an MOI study and demonstrated that sample number of $n \geq 97$ produced low sampling bias. In the present study, a total of 200 microscopically-confirmed knowlesi human blood samples (100 from Peninsular Malaysia and 100 from Malaysia Borneo) were obtained from hospitals, district health offices, state vector laboratories, and public health laboratories. Nested PCR targeting the 18S rRNA gene (Snounou *et al*., 1993; Imwong *et al*., 2009) was conducted to re-confirm the 200 samples for *P. knowlesi* infection.

DNA extraction

One hundred µl of whole blood was used to extract the genomic DNA using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol.

Genotyping of *Pkmsp-1* block IV

Oligonucleotide primers (Table 1) were designed for the nested PCR amplification of the two allelic families within block IV of *Pkmsp-1* (Noordin *et al*., 2023). The primer pairs for the primary amplification flanked the block IV region. Allele-specific primer pairs targeting nucleotide regions that distinguished the two allelic families within block IV were designed for the secondary amplification. The primer binding regions are illustrated in Figure 1.

A total of 200 microscopy and PCR-confirmed *P. knowlesi* samples (100 from Peninsular Malaysia and 100 from Malaysian Borneo) were used for the MOI study. All PCR reagents were from Promega, Wisconsin, USA. The 25 µl amplification reaction consisted of 1X Green GoTaq® Reaction Buffer, 4 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of each primer, and 1.0 U of GoTaq® G2 DNA Polymerase. For each sample, 0.5 µl of template DNA was used. The primary amplification was conducted with an initial denaturation at 94°C for 3 mins followed by 30 cycles of denaturation at 94°C for 30 secs, annealing at 58°C for 1 min, and extension at 72°C for 1 min. For the secondary amplification, annealing temperature was set at 60°C for 1 min and extension at 72°C for 30 secs. The final extension was carried out at 72°C for 5 mins for both rounds of amplification. The amplicons were then analysed on a 3% agarose gel pre-stained with SYBR® Safe DNA gel stain (Invitrogen, Eugene, USA) at 80 V for 60 mins using 1X TBE buffer and visualised using Gel Doc XR+ System (Bio-Rad, California, USA). The Image Lab version 6.0 software was used to estimate the the size of the amplicons by referring to the Bio-Rad 100 bp Molecular Ruler as the standard. The amplicons were then grouped manually into 25 bp "bins" and isolates within the same bin were considered as the same allelic variant/genotype.

Confirmation of allelic variants

A total of 9 amplicons, consisting of Type 1 (n=4) and Type 2 (n=5) amplicons, were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol, and the purified amplicons were ligated into the pGEM-T® TA cloning vector (Promega, Wisconsin, USA) based on 3:1 insert-vector ratio. The ligation products were then transformed into the One Shot™ *Escherichia coli* TOP10F' competent cells (Invitrogen, Eugene, USA). After a 16-hour incubation, colony PCR was performed, and the amplicons were analysed using agarose gel electrophoresis as mentioned before but using 1% agarose gel. The plasmids containing the *Pkmsp-1* insert were harvested from positive recombinant clones using QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and sent to a commercial laboratory (First BASE Laboratories Sdn. Bhd., Malaysia) for nucleotide sequencing (Sanger method). The raw sequences were trimmed using Bioedit ver 7.2 software and subjected to nBLAST to confirm the identity of the sequences. The sequencing step also further adjusted and verified the estimation of amplicon size retrieved using the Image Lab ver 6.0 software.

Calculation of MOI

The mean MOI by dividing the total number of amplicons detected for both allelic families with the total number of positive isolates as shown in the formula below:

$$Mean\ MOI = \frac{Total\ number\ of\ amplicons\ detected}{Total\ number\ of\ positive\ isolates}$$

Isolates with one detected allele were categorised as having monoclonal infection, while isolates with more than one detected allele (intra- or inter-allelic families) were categorised as having polyclonal infection.

RESULTS

MOI of *P. knowlesi* in Malaysia

The allele-specific primer pairs in the secondary amplification were able to produce amplicons for 188 out of the 200 blood samples: 94 samples per region (success rate: 94%). Samples that failed to be amplified were of low parasitaemia level. For the calculation of mean MOI, the number of isolates for each region (i.e., Peninsular Malaysia, Malaysian Borneo) was equilibrated at 100 per region. The size range of amplicons for T1 and T2 was 225-350 bp and 250-350 bp, respectively. The size of each amplicon has been deducted

Table 1. Primer sequences for nested PCR to genotype *P. knowlesi* based on the polymorphic block IV of the *PkMSP-1* gene

Amplification	Allelic family	Primer	Sequence 5' to 3'	Expected size
Primary	*	N1_F N1_R	GGAGCAAGTGAAACAGCACCGTTCAGGGTTCATGGTGGAG	500 – 600 bp
Secondary	Type 1	Type-1_F Type-1_R	CAGCACAAGCAGCATCACCGGGGCTGCTGCTGCTGGG	200 – 350 bp
	Type 2	Type-2_F Type-2_R	GCAACAACAGCTGTACAATTGTTTCTGTAGGCGTTGC	250 – 350 bp

* Primer pairs target the conserved region, hence no allelic family present.

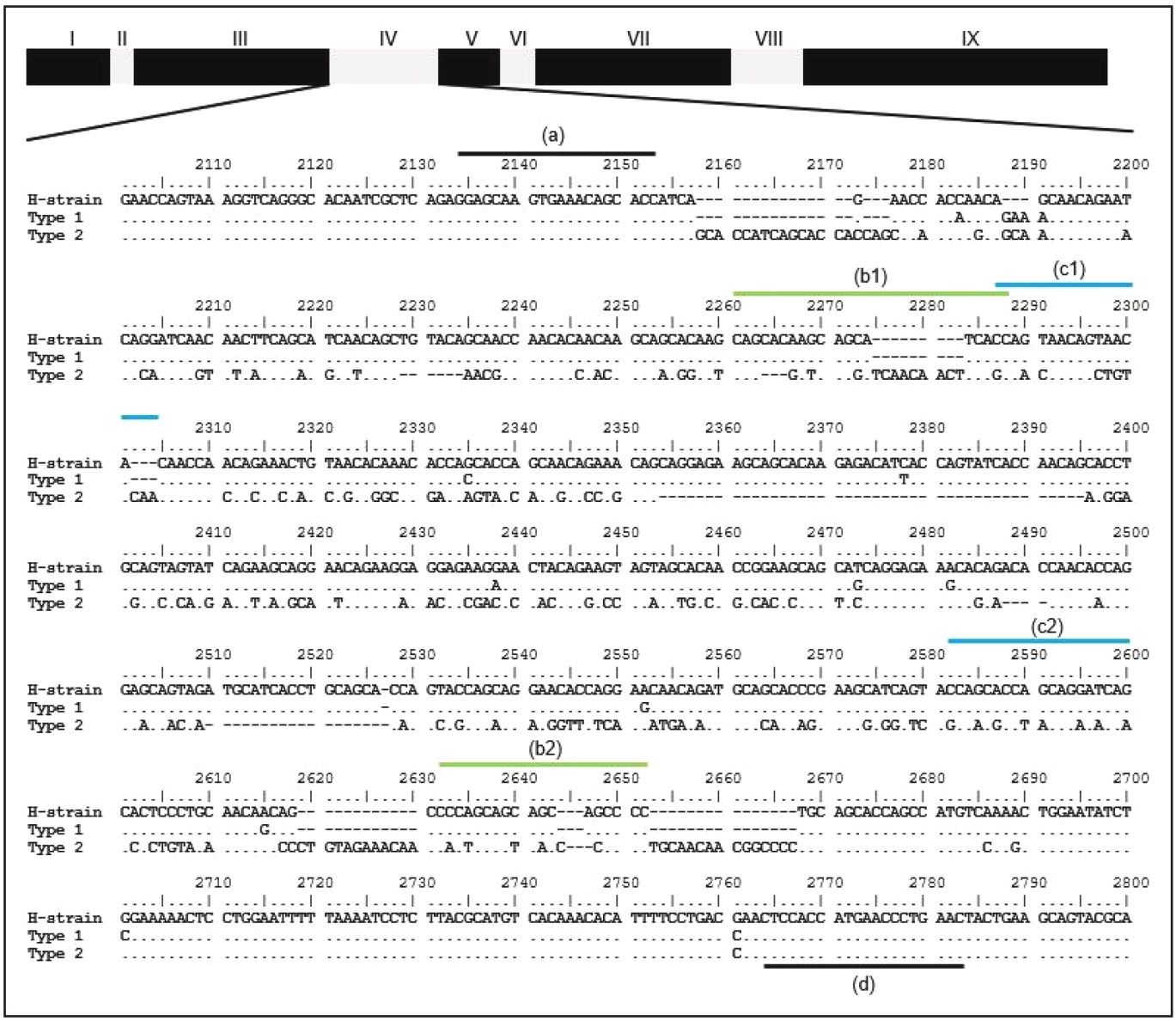

Figure 1. Schematic diagram of nested PCR primer annealing sites in the *Pkmsp-1*. Black boxes indicate conserved blocks while the grey boxes indicate polymorphic blocks. (a) and (d) are the binding sites for the primary amplification of block IV. (b1) and (b2) are the primer binding sites targetting the Type 1 allelic family. (c1) and (c2) are the primer binding sites for the Type 2 allelic family.

Table 2. Genetic diversity of *P. knowlesi* Malaysian isolates based on *Pkmsp-1* block IV

Origin of isolates	Allele type	Allelic frequency (%)	Total amplicon detected (n)	Monoclonal infection (%)	Polyclonal infection (%)	Mean MOI
Peninsular Malaysia	T1	47	79	67	33	1.40
	T2	25	61			
	T1 + T2	28				
Malaysian Borneo	T1	13	63	48	52	1.58
	T2	40	95			
	T1 + T2	47				

by 9.2 bp to accommodate the average base pair difference between Sanger sequencing and the Image Lab ver 6.0 software. Approximately half of the samples from Malaysian Borneo (52%) were noted to harbour polyclonal infection, which was higher than the samples from Peninsular Malaysia (33%) (Table 2). The mean MOI for Malaysian Borneo was slightly higher as compared to Peninsular Malaysia (1.58 *vs* 1.40). Despite having a lower percentage of polyclonal infection, the mean MOI in Peninsular Malaysia did not differ greatly from that of Malaysian Borneo. This is due to the relatively high proportion of polyclonal infections in Peninsular Malaysia samples having more than two alleles. Biased distribution of allelic family was observed where the T1 allelic family was more prevalent in Peninsular Malaysia (n = 75) than in Malaysian Borneo (n = 60). T2 allelic family, on the other hand, was more prevalent in the Malaysian Borneo (n = 87) than in Peninsular Malaysia (n = 53).

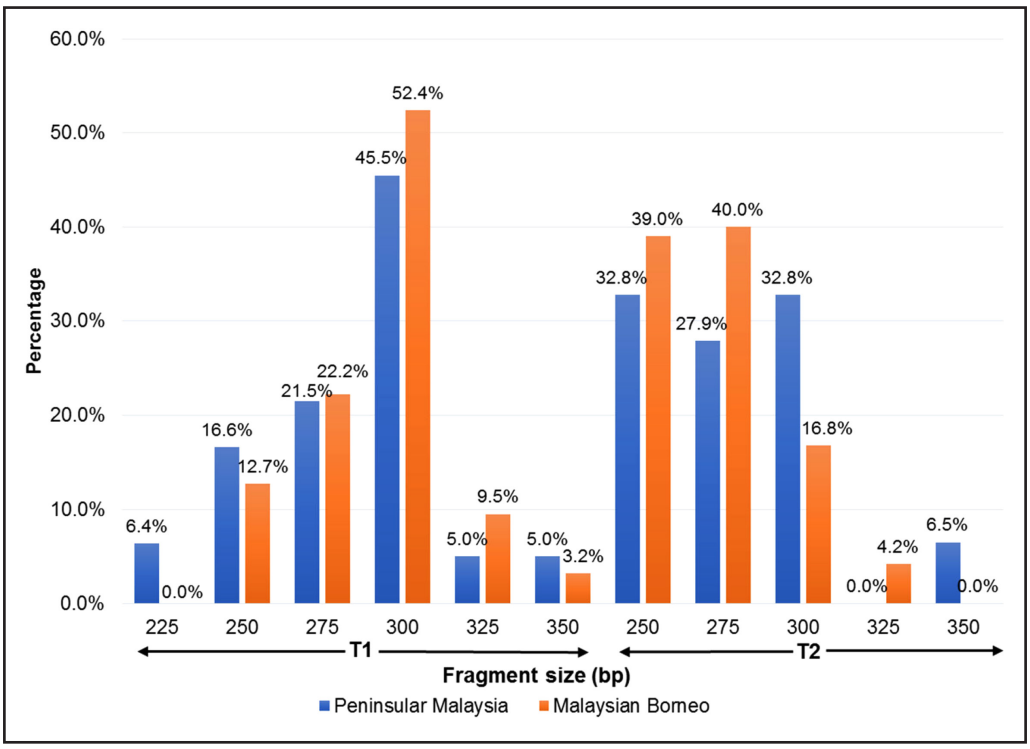

Figure 2. Prevalence of T1 and T2 alleles based on the amplicon size (bp) with the "bins" width set at 25 bp.

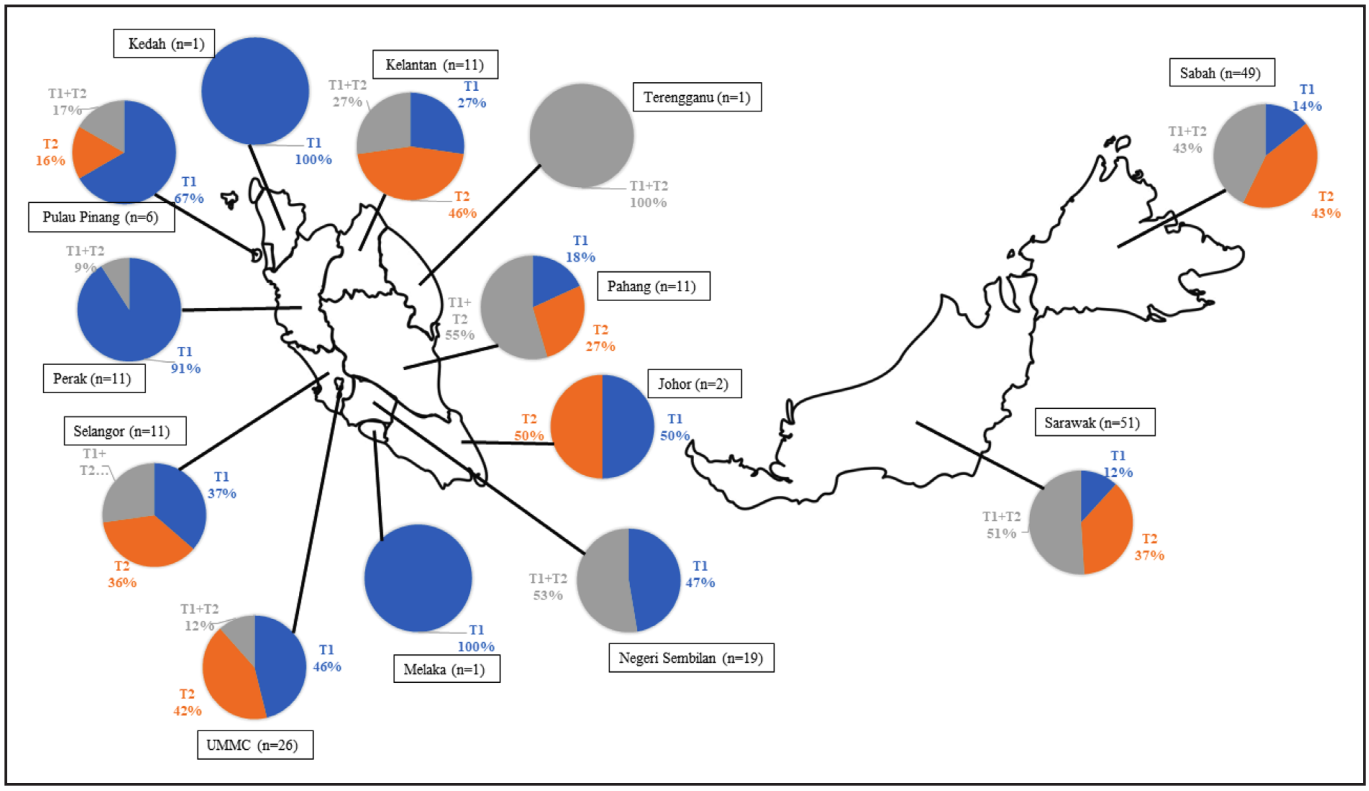

Figure 3. Distribution of T1 and T2 allelic families in Peninsular Malaysia and Malaysian Borneo.

Allelic distribution of *Pkmsp-1* block IV

Figure 2 presents the prevalence of alleles according to amplicon size. Six alleles were detected in the T1 family (225-350 bp). All six T1 alleles were detected in Peninsular Malaysia as opposed to five in Malaysian Borneo (250-350 bp). Five alleles were detected in the T2 family (250-350 bp). Four T2 alleles (250, 275, 300, 350 bp) were detected in Peninsular Malaysia, and four also (250, 275, 300, 325 bp) were detected in Malaysian Borneo. In the T1 allelic family, the 300 bp allele was the predominant allele in both Peninsular Malaysia (45.5%) and Malaysian Borneo (52.4%), while the 250 bp and 300 bp alleles were the predominant alleles of the T2 family in Peninsular Malaysia (32.8%). The predominant allele of the T2 family in Malaysian Borneo was 275 bp (40.0%). The detailed distribution of alleles in Peninsular Malaysia and Malaysian Borneo is illustrated in Figure 3.

DISCUSSION

This is the first report on the use of *Pkmsp-1* block IV in determining the MOI of *P. knowlesi*. Malaysian Borneo exhibited a slightly higher mean MOI than Peninsular Malaysia. This is corroborated by the higher prevalence of polyclonal infection (52%) in Malaysian Borneo as compared to Peninsular Malaysia (33%). In endemic falciparum malaria settings, polyclonal infection is more common in areas with high transmission (Ferreira *et al*.,1998; Ekala *et al*., 2002) and also associated with severe forms of infection (Jamil *et al*., 2021). Environmental factors *viz* changes in land use and deforestation that lead to complex changes between the hosts and the vector are among the predisposing factors to higher cases of *P. knowlesi* (Davidson *et al*., 2019). Undoubtedly, human activities which include working in the vicinity of a forest, entering the forest at dusk, and having a macaque as a pet are associated with acquiring knowlesi infection (Chin *et al*., 2021).

The *P. falciparum PfMSP-1* block II has been exploited as the genotyping marker for determining the MOI in a wide range of endemicity. The marker has also been proven to be useful as a transmission intensity predictor for seasonal variation (Bruce *et al*., 2011; Sondo *et al*., 2020; Baina *et al*., 2023). It has been observed previously that the genetic diversity indices along with the mean MOI were low in western Ethiopia following effective malaria intervention programs (Tadele *et al*., 2022). At this juncture, however, the mean MOI (Peninsular Malaysia: 1.40, Malaysian Borneo: 1.58) obtained in the present study can yet to be used in the classification of the transmission intensity of *P. knowlesi.* Further studies are deemed necessary to demonstrate this. Nonetheless, we envisage the use of *Pkmsp-1* block IV-based MOI as indicator for transmission intensity as well as an index for assessing efficacy of interventional or control programs for knowlesi malaria.

Multiple inoculations by several infected Anopheline mosquitoes as well as genetically diverse sporozoites inoculated in a single blood meal have been suggested to be the causes of high MOI. Meiotic recombination and mutation of the plasmodial parasites following several rounds of DNA replication cycles presumably will sustain the diversity of the parasites through positive selection and disseminates these alleles within a population. Subsequently, this increases the parasites' multiplicity/complexity within a locality – leading to high MOIs. High MOIs were evident previously in high-intensity transmission areas (Mwingira *et al*., 2011; Tusting *et al*., 2014; Abukari *et al*., 2019; Sondo *et al*., 2020) and this should prompt the authorities to either scale up the intervention and/or to strategise transmission control activities.

In this study, the agarose gel electrophoresis coupled with the Image Lab version 6.0 software programme was used to detect the amplicons and determine their sizes. This combined approach is inexpensive and easy to perform. However, but it is not be able distinguish amplicons of almost similar sizes (i.e., different alleles), and therefore may cause underestimation of allele frequency and MOI. This can be overcome by the use of capillary electrophoresis, which has higher resolution power to distinguish amplicons with size difference of only a few base pairs. Although the cost of performing capillary electrophoresis is comparatively higher, nonetheless it is worthwhile to employ this approach in future studies in order to attain more precise estimates of *Pkmsp-1* block IV-based MOI.

CONCLUSIONS

Our study is the first to report the use of *Pkmsp-1* as a genotyping marker for MOI of human knowlesi malaria. This approach provides an alternative and yet simpler method relative to microsatellite genotyping in assessing the transmission intensity of *P. knowlesi* in a population. Since this study used human samples only, future studies should focus on samples from the macaque populations as macaques are the natural hosts of *P. knowlesi*.

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Conflict of interest

The authors declare no conflict of interest.

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